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Author: Polliane Morais de Carvalho Maria Beatriz Duarte Gavião Guy Howard Carpenter

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Altered autophagy and sympathetic innervation in salivary glands from high-fat diet mice

Polliane Morais de Carvalho [a]

Maria Beatriz Duarte Gavião [b]

Guy Howard Carpenter [a]

[a] Salivary research, Department of Mucosal and Salivary Biology, King's College London Dental Institute, London SE1 9RT, United Kingdom

[b] Department of Pediatric Dentistry - Piracicaba Dental School - University of Campinas (UNICAMP), Av Limeira 901, Piracicaba, SP 13414-903, Piracicaba, SP, Brazil

*Corresponding author:

Dr Polliane M Carvalho

Department of Mucosal and Salivary Biology

King's College London Dental Institute,

Tower Wing Guy's, Hospital

London SE1 9RT, United Kingdom

Email: pollianecarvalho@gmail.com

Highlights:

- Adiponectin levels decreased in the High Fat Diet group after three months
- Autophagy increased after three months of High Fat Diet
- mTOR showed no activation regarding the time point studied
- Tyrosine hydroxylase decreased after two and three month of High Fat Diet

Abstract

Objective: to investigate the effects of a high fat diet (HFD) on salivary glands in vivo, in a mouse model. In particular, whether it will induce the appearance of fat cells in salivary glands, alterations related to autophagy, mTOR pathway and sympathetic innervation. **Design:** 27 adult female ICR mice were separated in six groups. Three groups fed with (HFD) containing 55% fat, for one, two and three month and another three groups fed with normal diet (2.7% of fat), for the same time periods. The submandibular glands and liver were dissected and part homogenized for protein analyses and part fixed in formalin for histological analyses **Results:** After three months the HFD fed mice total body weight fold change increased compared to controls. The Oil Red O staining showed no fat cells deposit in salivary gland however a large increase was observed in liver after three months of HFD. Adiponectin levels were significantly decreased in the HFD group after three months. The group fed with HFD for three months showed increased conversion of the LC3 autophagy marker in salivary gland. mTOR showed no activation regarding the time point studied. Tyrosine hydroxylase significantly decreased after two and three month of HFD. **Conclusion:** HFD caused several changes after three months however the earliest change was noticed after two months regarding sympathetic innervation. This suggests neural alteration may drive other diet induced changes in salivary glands. These early changes may be the starting point for longer term alterations of salivary glands with alterations in diet.

Keywords: submandibular gland, high fat diet, autophagy, mTOR, sympathetic innervation

Introduction

Salivary glands are well known to change aspects of structure according to diet. Neural inputs from taste and chewing maintain the salivary parenchyma in a normal healthy state. Moreover the parenchyma of salivary glands are subjected to other influences such as hormones that affect changes in its morphological characteristics leading to local or systemic alterations.[1] One such change is adipose tissue infiltration between acini parenchyma associated with conditions like: Human Imunodeficiency virus (HIV), [2] hypertriglyceridemia, [3] but most commonly with ageing in the absence of any obvious disease process [4, 5].

White adipose tissue stores excess calories and prevents the toxic accumulation of excessive nutrients in non-adipose tissue. [6] It also communicates with organs by producing adipokines as part of an endocrine system. Adiponectin is an adipokine involved in the regulation of energy metabolism and inflammatory responses. [7] It is considered an important protective adipokine against obesity. Previous data has shown that adiponectin expression and its receptors are detectable in salivary gland even in the absence of fat cells. [8, 9] Thus receptors present in salivary glands might be involved in the effects of metabolic changes regulation such as local inflammatory and immunological processes. [10]

The consumption of high caloric palatable foods has increased significantly in recent decades leading obesity to achieve epidemic proportions with negative impacts for the economy and general health worldwide. [11, 12] Therefore understanding the pathophysiology mechanism involved in the development of obesity and its related diseases has become timely. Previous studies have pointed out an association between obesity and oral pathologies such as periodontitis, [13-15] but the changes that obesity and its molecular pathway induce in salivary glands are poorly understood. Recently, it has been demonstrated that obesity and its related metabolic comorbidities are closely associated with enhanced autophagy and up regulation of some markers such as LC3 in adipose tissue. [16,17]

It is likely that diet affects salivary gland via several potential mechanisms. Excessive nutrient intake promotes signalling through the mammalian target of rapamycin (mTOR), which in turn may lead to alterations in cellular metabolic signalling, insulin resistance and obesity related diseases. [18, 19] In most normal healthy tissues such as liver and muscle, mTOR is active and drives protein synthesis. In salivary glands it is normally inactive but becomes activated with atrophy [20,21] However the effect of diet on salivary glands regarding mTOR pathway has not been elucidated.

The parotid and submandibular glands of rat, mouse and human receive parasympathetic and sympathetic innervations that regulate fluid, ion and protein secretion into saliva. [22] Tyrosine hydroxylase (TH) catalyses the reaction in which L-tyrosine is hydroxylated to L-Dopa a precursor of dopamine and therefore a useful marker of sympathetic nerves. Dysregulation of dopamine has also been implicated in the development of obesity.[23] Since white adipose tissue is innervated by sympathetic nervous system [24] and tyrosine hydroxylase mRNA expression in neuronal tissue can be regulated by macronutrient intake [11] it seemed reasonable to assess sympathetic nerves in salivary gland.

Furthermore the formation of adipose tissue in salivary glands remain an enigma, it is uncertain if their appearance is related to obesity or HFD. Since adipose cells release adipokines and salivary cells have adipokines receptors their influence needs to be elucidated. Thus the objective of this research was to investigate the effects of a high fat diet on salivary glands and determine if this will induce fat cells or alter autophagy, mTOR activity or sympathetic innervation.

Material and Methods

A total of 27 adult female ICR mice were obtained from Charles Rivers Laboratories (Margate, UK); weighing an average of 22-30g upon arrival. Mice were housed with food and water provided *ad libitum*. A 12h light-dark cycle was maintained at a constant temperature of 20-22°C. Environmental enrichments (tunnels and nesting material) were provided in each cage. Animals were allowed to acclimatize to their new environmental for one week before experimental procedures. All procedures in the animals were conducted in accordance with UK Home Office Animal (Scientific Procedures) Act 1986.

A group of five mice were fed on a high fat diet (HFD) for one month (HFD1) (but one was excluded due to aggressive behaviour), a group of five mice were fed on HFD for two month (HFD2), and another group of five fed on high fat diet for 3 month (HFD3). The high fat diet was providing by Special Diet Services (SDS) UK and has 55% (w/v) of fat. The controls groups comprised of four mice for each time point studied and were fed standard pelleted food (SDS UK) containing 2.7% of fatty acid added to its composition. Animal body weights were recorded daily. Mice were sacrificed by an overdose of pentobarbitone.

After the time points described the submandibular gland and liver samples were removed. Submandibular glands in the HFD group were increasingly surrounded by fat tissue compared to controls but were separated from sublingual glands and weighed. The tissues were then fixed in 4% formalin or frozen in liquid nitrogen for biochemical analyses.

Histochemical staining of tissue samples: Submandibular glands and liver were embedded in wax and 5 µm thick sections were cut and mounted on super-frost plus-coated slides. General morphology of the tissue sections was assessed by H&E staining. Fat deposits were investigated by Oil Red O staining; frozen sections were fixed in 10% formaldehyde, washed with distilled water, rinse with 60% isopropanol, stained with Oil Red O (0.18% w/v in isopropanol) for 15 minutes, rinsed with 60% isopropanol and finally counter-stained with haematoxylin for the detection of nuclei.

Immunohistochemistry on tissue sections

The tissue sections were first de-waxed and then incubated for ten minutes in a solution of 3% hydrogen peroxide to inhibit the endogenous peroxidase. Sections were incubated at 100°C for 15 min in citric acid buffer (pH 6.0) in a pressure cooker base. Sections were covered with blocking solution (2% BSA in 1x TBS and azide, pH7.6) and incubated for 5 minutes for further application of primary antibodies. The antibodies were incubated overnight at room temperature. The next day the sections were washed for 10 mins in 500ml 1x TBS, with stirring and after the secondary antibody was applied. The peroxidase activity was visualized with diaminobenzidine tetra-hydrochloride (DAB) (0.5mg/ml) and counterstained with Mayer's Haematoxylin.

Tissue Preparation and immunoblotting

Submandibular tissue specimens stored at -80°C were homogenized in 19 volumes (w/v) of ice-cold homogenization buffer 1% Triton X-100, Tris-HCL pH 7.4, 1mM EDTA, and a 1% v/v dilution of protease cocktail set 1 (Merck Chemicals Ltd, Nottingham, UK) using an Ultra-Thurrax homogenizer (IKA Labortechnik, Staufen, Germany).

SDS- PAGE of samples was carried out (NUPAGE Novex Bis-Tris 4-12% gel; Life technologies, Paisley, UK). Proteins resolved by electrophoresis were then electroblotted onto 0.45 μm nitrocellulose membranes (Anderman and Co., Kingston-Upon-Thames, UK). The procedure followed for immunoblotting is that which has been used previously. [25] Membranes were imaged in a ChemiDoc Imaging System (BIORAD Laboratories Ltd, Hertfordshire, UK), with optimized exposure times and the built in high sensitivity blot detection which highlights over saturated pixels to obtain ideal exposure of images of the protein bands. Band intensity from immunoblots were quantified using the image analysis software image version 1.46 (NIH, Maryland, MD, USA). Blots were reprobed for beta actin to normalize protein loading between lanes thus each bar represents the mean normalized to β-actin ± S.E.M.

Antibodies

The antibody adiponectin (1:2000 for western blotting) was obtained from Sigma-Aldrich (Poole, Dorset, UK).The antibodies: LC3A/B (1:1000 for western blotting), anti-phospho-4E-BP1 (1:1000 for western blotting), anti-phospho-S6 ribossomal protein (1:1000 for western blotting), tyrosine hydroxylase (1:1000 for western blotting) were obtained from Cell Signalling Technology (Hertfordshire, UK). Tyrosine hydroxylase (1:1000 for immunohistochemistry) was obtained from Abcam (Cambridge, MA, USA) and anti-β-actin (1:1000) was from Sigma-Aldrich (Poole, Dorset, UK). Secondary antibodies included polyclonal goat anti-mouse immunoglobulin-HRP and polyclonal goat anti-rabbit immunoglobulin-HRP from Dako Ltd (Ely, UK).

Statistical analysis:

Data analysis was performed using the Graph Pad Prism 5 package (GraphPad Software Inc., La Jolla, CA). Results were expressed as means ± S.E.M., and were statistically compared by student's t-test, or ANOVA followed by Tukey test, $P<0.05$ was considered statistically significant.

Results:

1-Changes in body and salivary glands weights:

As expected, the studies groups submitted to high fat diet (HFD1, HFD2 and HFD3) showed significant increase in body weight when compared to the starting weight ($p<0.05$), although the controls also increased their body weight significantly during the same time points. However, the fold change increase (the ratio between the initial and final weight) when controls and HFD were compared showed statistically significant differences only after three months (control and HFD3 Fig 1A). Submandibular gland weights remained similar in all groups and when expressed as a ratio of body weight (Fig1B) significantly decreased in the HFD after three months.

2-Histological analysis:

The morphology of submandibular glands assessed by H&E did not change among groups. Both acinar and ductal cells appeared normal and fully replete with storage granules with few signs of inflammation. Figures 2A, 2B, 2C, and 2D represent respectively a control gland, HFD1, HFD2 and HFD3 submandibular glands. In contrast the liver showed several histological changes with HFD. H&E showed cytoplasmic storage vacuoles after 3 months (Figure 3A and 3B represent control and HFD3 liver) which the Oil Red O staining suggested were large accumulation of fat levels with lots of red droplets in group HFD3 (Figure 4C and 4D control and HFD3 liver tissue respectively). However the Oil Red O technique revealed no fat deposition in submandibular gland from controls group or HFD (figures 4A and 4B).

3-Adiponectin:

Measurements of adiponectin in submandibular homogenates were done by immunoblotting and HFD for one month and two months were not different from respective controls. However, the high fat diet group for three months had significantly decreased levels of adiponectin in submandibular glands compared to controls (Figure 5A and 5B).

4-LC3:

Tissue homogenates of submandibular glands were analysed for evidence of autophagy. The LC3 ratios measured by immunoblotting were significantly increased after three month compared with controls (Figures 6A and 6B).

5-mTOR status:

Evidence of mTOR activation was assessed by phosphorylated 4E-BP1 and phospho - S6 ribosomal protein. Immunoblotting revealed no activation of S6RP or phosphorylated 4E-BP1 in controls or HFD1, HFD2, and HFD3 groups (Fig 7).

6-Tyrosine hydroxylase:

In the first month the levels of TH measured in submandibular gland homogenate did not differ between controls and high fat diet fed ones (data not shown). However after two and three months the levels of TH decrease significantly in the groups fed with high fat diet. Figure 8A and 8C shows the normalized (to actin) ratio of TH expression between high fat diet and normal diet for two and three month respectively. The imunohistochemistry also shows decreased expression of TH in HFD fed ones after 3 months (Figures 9A and 9B) although it is not a quantitative method it does show staining localised to nerve-like structures.

Discussion

The adipogenic stimulus of a high fat diet caused a significant fold change in body weight only after three months when compared to a normal diet. Though the body weight of mice increased the submandibular glandular weight did not, suggesting fat tissue accounts for most of the increased body weight. Oil Red O staining also suggested no fat deposition in submandibular glands. Since obesity is characterized with enhanced lipid accumulation in the liver, [26] we checked the liver for fat deposition and indeed found a large increase in fat accumulation in the HFD after three months. Although 3 months is a short time for the HFD, and many other studies have used longer periods, 3 months was sufficient to affect body weight but not cause fat cell formation in the submandibular gland. Another study of C57BL/6 mice older than ninety weeks did not detect fat infiltration in submandibular gland [29]. Most previous reports of adipose tissue infiltration in human submandibular gland suggest ageing as the key factor rather than diet [27, 28]. However it cannot be excluded that the glandular tissue of mice are different from the human and may have some protective mechanism against adipose tissue infiltration.

Circulating adiponectin can regulate the function of several tissues. Ding et al (2013) [10] described perfusion of isolated salivary glands with adiponectin which stimulated saliva secretion. Therefore we investigated if a high fat diet could modify the levels of adiponectin in submandibular gland. Our results showed that after three months of a high fat diet the levels of adiponectin in submandibular glands significantly decreased. This result indicates that high fat diets and weight gain can possibly modify glandular responses which could lead to alterations in oral health. There is already substantial evidence for an association of obesity and periodontitis an inflammatory disease. Although we did not collect saliva there were no indications of hyposecretion (eg dry mouth, tongue atrophy, decreased body weight, reduced grooming etc.,) and indeed there are no reported associations between obesity and salivary hypofunction in the literature.

Autophagy is a homeostatic process that is constitutively active in essentially all tissues by removing damaged or misfolded proteins. In this study increased levels of autophagy as determined by LC3 ratios (LC3II/LC3I) occurred after three months of HFD compared to controls in submandibular gland. Previous studies have demonstrated that autophagy is up-regulated in adipose tissue of leptin-receptor deficient db/db mice.[30] Morgan-Bathke et al., (2014), showed that in submandibular gland autophagy could play a beneficial role in re-establishing salivary gland homeostasis following targeted head and neck radiation [31]. However in submandibular gland increased autophagy is associated with ligation – induced atrophic degeneration. [21] However there were no obvious histological signs of atrophy and most likely the increased autophagy is due to increased processing of lipids.

In nutrient abundant circumstances, mTOR signalling stimulates adipose tissue expansion. [32] Since the appearance of fat cells is common in human salivary glands we considered if mTOR pathway had become activated during the three months of HFD. In healthy adult mouse salivary gland mTOR is not active and did not become activated within three month of HFD. Our previous data showed activation of mTOR occurs in submandibular glands undergoing atrophy. [20] However this study underlines the complexity of the in vivo regulation of mTOR in salivary glands as other tissues such as adipose do become activated by HFD.

Fat cells have sympathetic innervation [24] and salivary glands (but not sublingual) have a dense sympathetic innervation. We used TH to study any changes to sympathetic innervation in mice on a HFD. Previous data showed that TH knockout mice presented hypophagia [33] and in brain tissue its expression is affected by diet. [11] Our results showed that the diet can significantly alter the protein expression after two months in submandibular glands. Although not conclusive this might indicate alterations in sympathetic innervations in the gland tissue. Adrenergic signalling from sympathetic nerves leads to an augmentation of protein secretion by parotid and submandibular glands. [34] It would be of interest to study protein secretion into saliva in response to autonomimetic agents such isoprenaline, as we have done previously.

A number of methodological issues limit generalizations regarding our results. The sample size was small, the duration of the HFD was short, differences between males and females fat regulation were not considered, nor was salivary flow measured and only the submandibular glands were examined. Thus additional larger scale study is important to validate our findings.

Conclusion

High fat diet induced weight gain significantly after three months which were associated with changes in submandibular glands. There were changes related mostly to sympathetic nerve changes. After three month of HFD the levels of adiponectin in submandibular gland were significantly reduced and autophagy seems to be increased. There was no obvious formation of fat accumulation in submandibular gland despite large increases in liver. Whether longer periods of HFD may induce atrophic changes remains to be determined.

Acknowledgments

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References:

1. Garcia DS, Bussoloti Filho I Braz J Otorhinolaryngol. 2013 Mar-Apr;79(2):173-6.Fat deposition of parotid glands

2. Kabenge C, Ng S, Muyinda Z, Ameda F. Diagnostic ultrasound patterns of parotid glands in human immunodeficiency virus-positive patients in Mulago, Uganda. Dentomaxillofac Radiol. 2010 Oct;39(7):389-99.

3. Sheikh JS, Sharma M, Kunath A, Fritz DA, Glueck CJ, Hess EV J Rheumatol. Reversible parotid enlargement and pseudo-Sjögren's syndrome secondary to hypertriglyceridemia.1996 Jul;23(7):1288-91.

4. Nagler RM, Hershkovich O. Relationships between age, drugs, oral sensorial complaints and salivary profile. Arch Oral Biol. 2005 Jan;50(1):7-16.

5. Kim SK. Changes in the secretory acinar cells of the rat parotid gland during aging. Anat Rec. 1984 Jul;209(3):345-54.

6. Lelliott C, Vidal-Puig AJ. Lipotoxicity, an imbalance between lipogenesis de novo and fatty acid oxidation. Int J Obes Relat Metab Disord.2004 Dec;28 Suppl 4:S22-8.

7. Borcherding DC, Hugo ER, Idelman G, De Silva A, Richtand NW, Loftus J, Ben-Jonathan N Dopamine receptors in human adipocytes: expression and functions. PLoS One. 2011;6(9):e25537.

8. Katsiougiannis S, Kapsogeorgou EK, Manoussakis MN, Skopouli FN (2006) Salivary gland epithelial cells: a new source of the immunoregulatory hormone adiponectin. Arthritis Rheum 54: 2295–2299.

9. Zhou Y, Sun X, Jin L, Stringfield T, Lin L, et al. (2005) Expression profiles of adiponectin receptors in mouse embryos. Gene Expr Patterns 5: 711–715.

10. Ding C, Li L, Su YC, Xiang RL, Cong X, Yu HK, Li SL, Wu LL, Yu GY. Adiponectin increases secretion of rat submandibular gland via adiponectin receptors-mediated AMPK signaling. PLoS One. 2013 May 7;8(5):e63878.

11. Li Y, South T, Han M, Chen J, Wang R, Huang XF. High-fat diet decreases tyrosine hydroxylase mRNA expression irrespective of obesity susceptibility in mice Brain Res. 2009 May 1;1268:181-9.

12. Lamy E, Simões C, Rodrigues L, Costa AR, Vitorino R, Amado F, Antunes C, do Carmo I. Changes in the salivary protein profile of morbidly obese women either previously subjected to bariatric surgery or not. J Physiol Biochem. 2015 Dec;71(4):691-702.

13. Nascimento GG, Leite FR, Correa MB, Horta BL, Peres MA, Demarco FF. Relationship between periodontal disease and obesity: the role of life-course events. Braz Dent J. 2014; 25(2): 87-9.

14. Alswat K, Mohamed WS, Wahab MA, Aboelil AA. The Association Between Body Mass Index and Dental Caries: Cross-Sectional Study.J Clin Med Res. 2016 Feb;8(2):147-52.

15. Prpić J, Kuis D, Glazar I, Ribarić SP. Association of obesity with periodontitis, tooth loss and oral hygiene in non-smoking adults. Cent Eur J Public Health. 2013; 21(4): 196-201.

16. Kovsan J, Blüher M, Tarnovscki T, Klöting N, Kirshtein B, Madar L, Shai I, Golan R, Harman-Boehm I, Schön MR, Greenberg AS, Elazar Z, Bashan N, Rudich A. Altered autophagy in human adipose tissues in obesity. J Clin Endocrinol Metab. 2011 Feb;96(2):E268-77.

17. Kosacka J, Kern M, Klöting N, Paeschke S, Rudich A, Haim Y, Gericke M, Serke H, Stumvoll M, Bechmann I, Nowicki M, Blüher M. Autophagy in adipose tissue of patients with obesity and type 2 diabetes. Mol Cell Endocrinol. 2015 Jul 5;409:21-32.

18. Jia G, Aroor AR, Martinez-Lemus LA, Sowers JR. Overnutrition, mTOR signaling, and cardiovascular diseases. Am J Physiol Regul Integr Comp Physiol. 2014; 307(10) :R1198-R1206.

19. Robinson KA, Hegyi K, Hannun YA, Buse MG, Sethi JK. Go-6976 Reverses Hyperglycemia-induced insulin resistance independently of cPKC. Inhibition in adipocytes. PLoS One. 2014; 9(10): e108963.

20. Bozorgi SS, Proctor GB, Carpenter GH. Rapamycin delays salivary gland atrophy following ductal ligation. Cell Death Dis. 2014 Mar 27; 5:e1146.

21. Silver N, Proctor GB, Arno M, Carpenter GH. Activation of mTOR coincides with autophagy during ligation-induced atrophy in the rat submandibular gland.Cell Death Dis. 2010;1:e14.

22. Proctor GB, Carpenter GH. Salivary secretion: mechanism and neural regulation. Monogr Oral Sci. 2014;24:14-29.

23. Wang, G.J., Volkow, N.D., Logan, J., Pappas, N.R., Wong, C.T., Zhu, W.,et al., 2001. Brain dopamine and obesity. The Lancet 357, 354–357.

24. Bartness TJ, Liu Y, Shrestha YB, Ryu V. Neural innervation of white adipose tissue and the control of lipolysis. Front Neuroendocrinol. 2014; 35(4): 473-93.

25. Carpenter GH, Proctor GB, Ebersole LE, Garrett JR. Secretion of IgA by rat parotid and submandibular cells in response to autonomimetic stimulation in vitro. Int Immunopharmacol. 2004 Aug; 4(8):1005-14.

26. Yang L, Li P, Fu S, Calay ES, Hotamisligil GS. Defective hepatic autophagy in obesity promotes ER stress and causes insulin resistance. Cell Metab. 2010 Jun 9;11(6):467-78.

27. Scott J. Quantitative age changes in the histological structure of human submandibular salivary glands. Achieves of oral Biology 1977; 22 (3):222-7.

28. Waterhouse JP, Chisholm DM, Winter RB, Patel M, Yale RS. Replacement of functional parenchymal cells by fat and connective tissue in human submandibular salivary glands: an age-related change. Journal of Oral Pathology 1973; 2(1):16-27.

29. Choi SS, Park J, Choi JH. PPARγ as a revisiting target for treatment of metabolic disorders. BMB Rep. 2014 Aug 25. pii: 2934.

30. Cinti S, Mitchell G, Barbatelli G, Murano I, Ceresi E, Faloia E, Wang S, Fortier M, Greenberg AS, Obin MS. Adipocyte death defines macrophage localization and function in adipose tissue of obese mice and humans. J Lipid Res. 2005 Nov;46(11):2347-55.

31. Morgan-Bathke M, Harris ZI, Arnett DG, Klein RR, Burd R, Ann DK, Limesand KH. The Rapalogue, CCI-779, improves salivary gland function following radiation. PLoS One. 2014 Dec 1;9(12):e113183.

32. Cai H, Dong LQ, Liu F· Recent Advances in Adipose mTOR Signaling and Function: Therapeutic Prospects.Trends Pharmacol Sci. 2016 Apr;37(4):303-17.

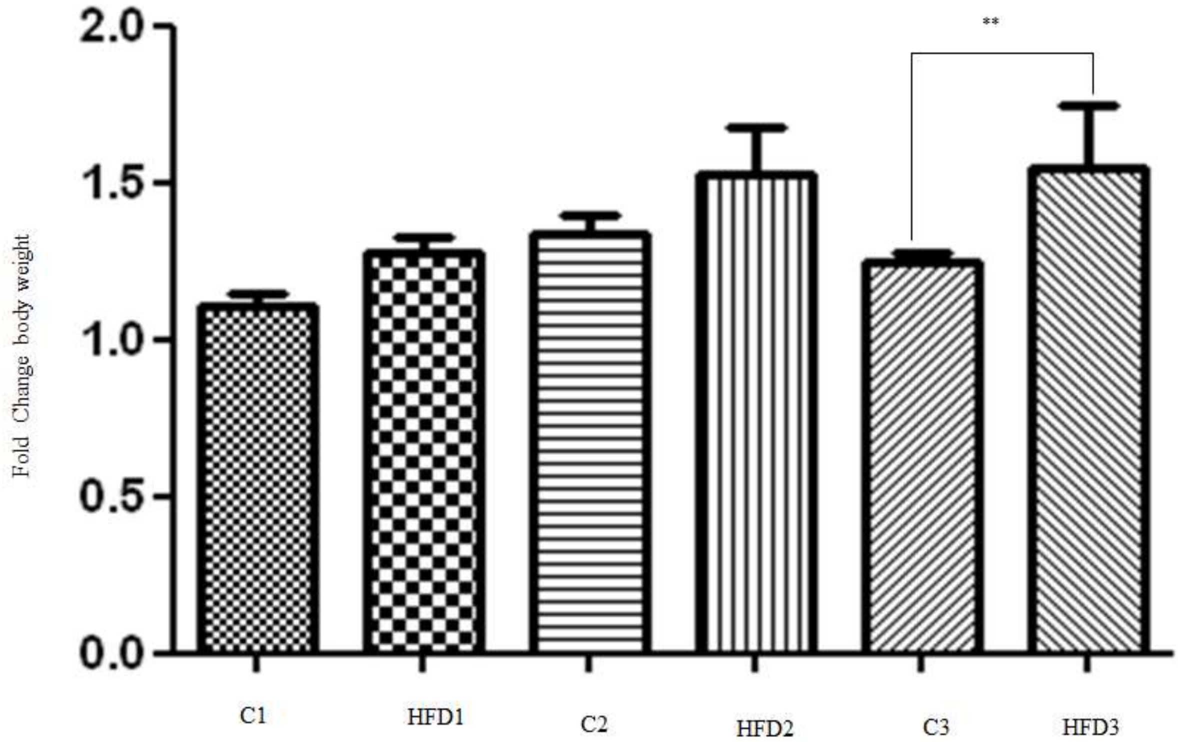
33. Zhou QY, Palmiter RD. Dopamine-deficient mice are severely hypoactive, adipsic, and aphagic.Cell. 1995 Dec 29;83(7):1197-209.

34. Proctor GB. The physiology of salivary secretion. Periodontol 2000. 2016 Feb;70(1):11-25.

Figure Legends:

Figure 1: (a) represents the fold change body weight comparing controls and HFD fed ones. (b) represents a ratio: submandibular gland weight divided by body weight.

Figure 1A

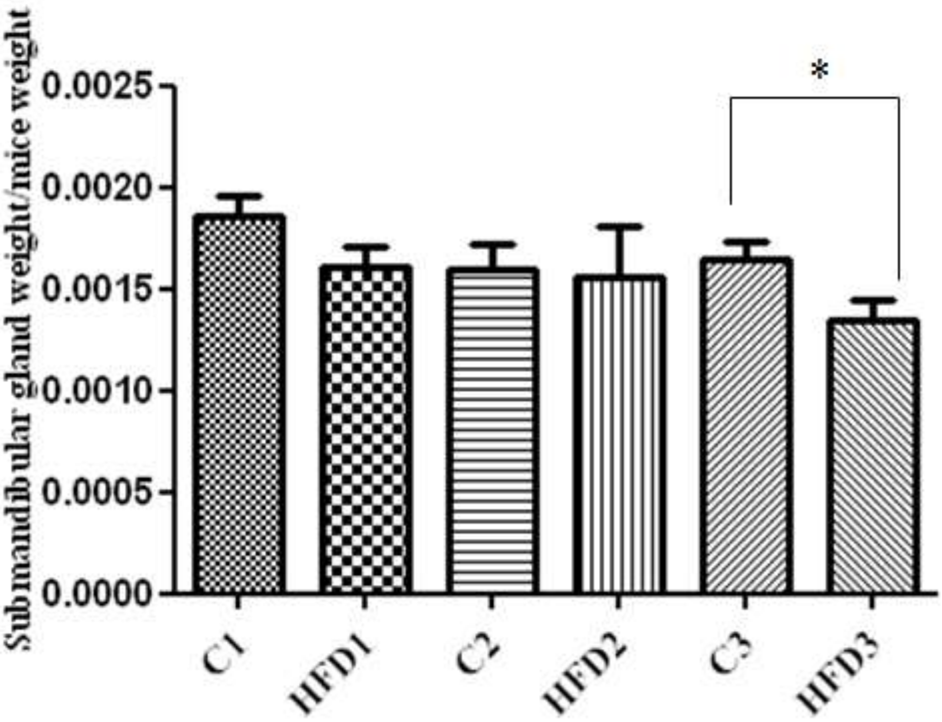


C1, C2, C3 controls fed on normal diet for 1, 2 and 3 month

HFD1, HFD2, HFD3 mice fed on high fat diet for 1, 2 and 3 month

* *P*<0.05 ANOVA

Figure 1B



C1, C2, C3 controls fed on normal diet for 1, 2 and 3 month

HFD1, HFD2, HFD3 mice fed on high fat diet for 1, 2 and 3 month

**P*<0.05 ANOVA

Figure 2: (a), (b), (c), and (d) represent Haematoxylin and Eosin (H&E) staining of submandibular gland respectively from a control, HFD1, HFD2 and HFD3 mice.

Figure 2A:

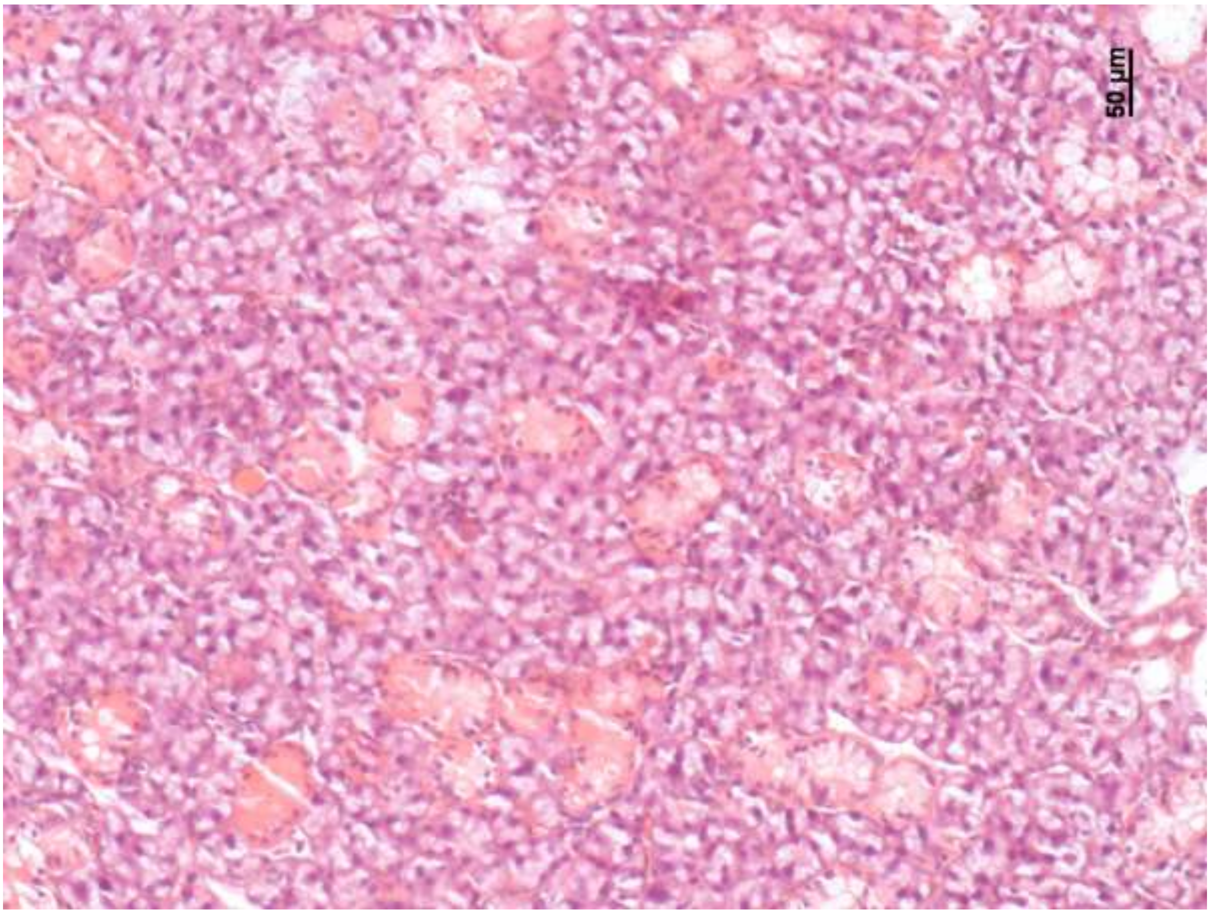


Figure 2B

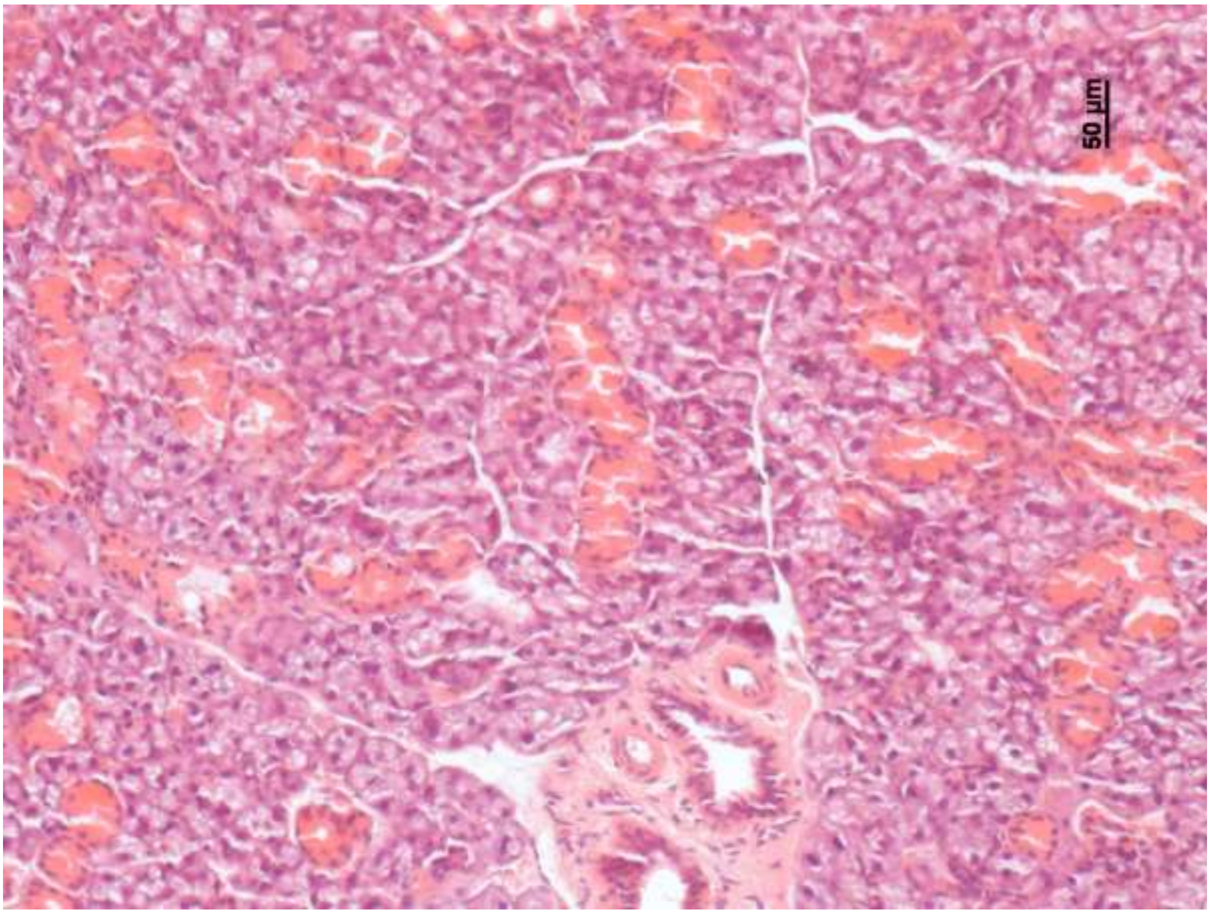


Figure 2C

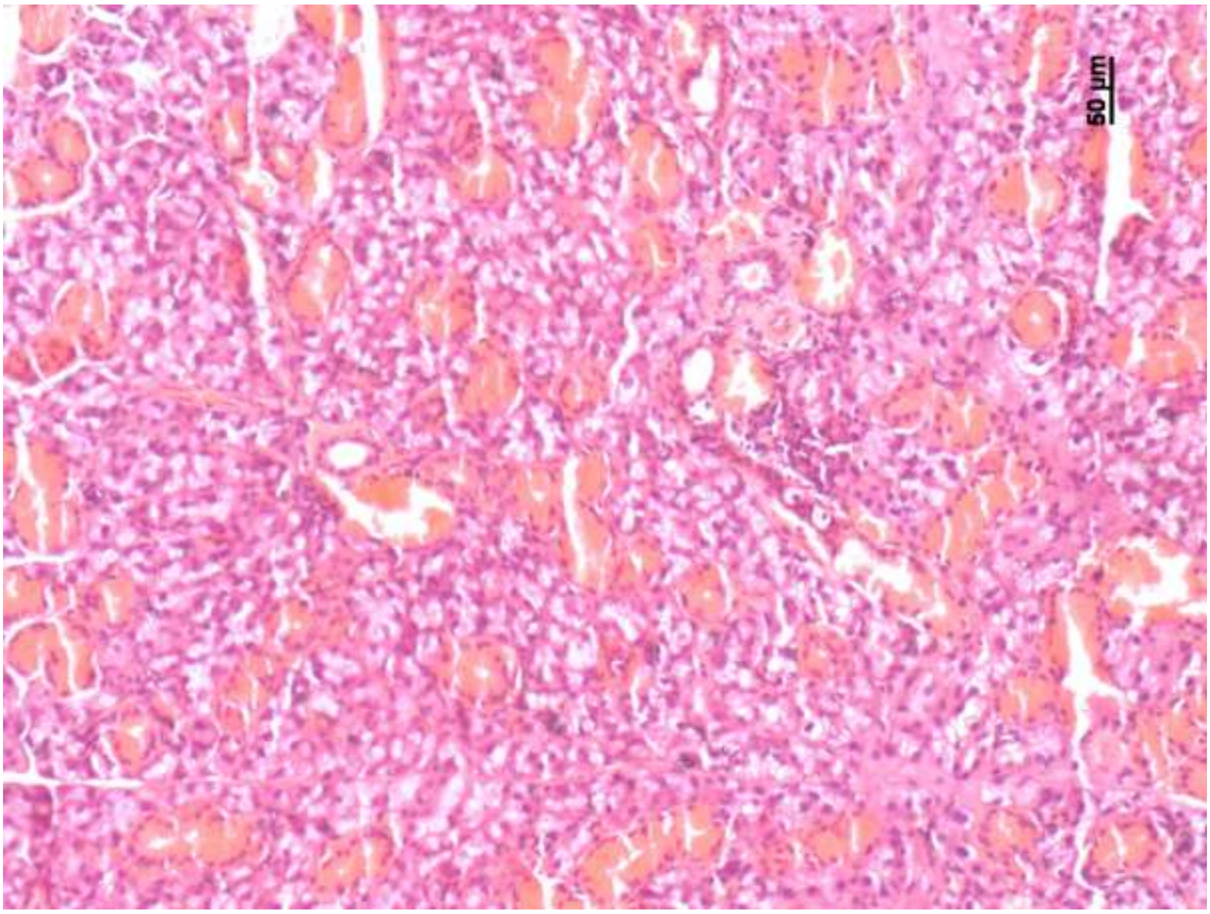


Figure 2D

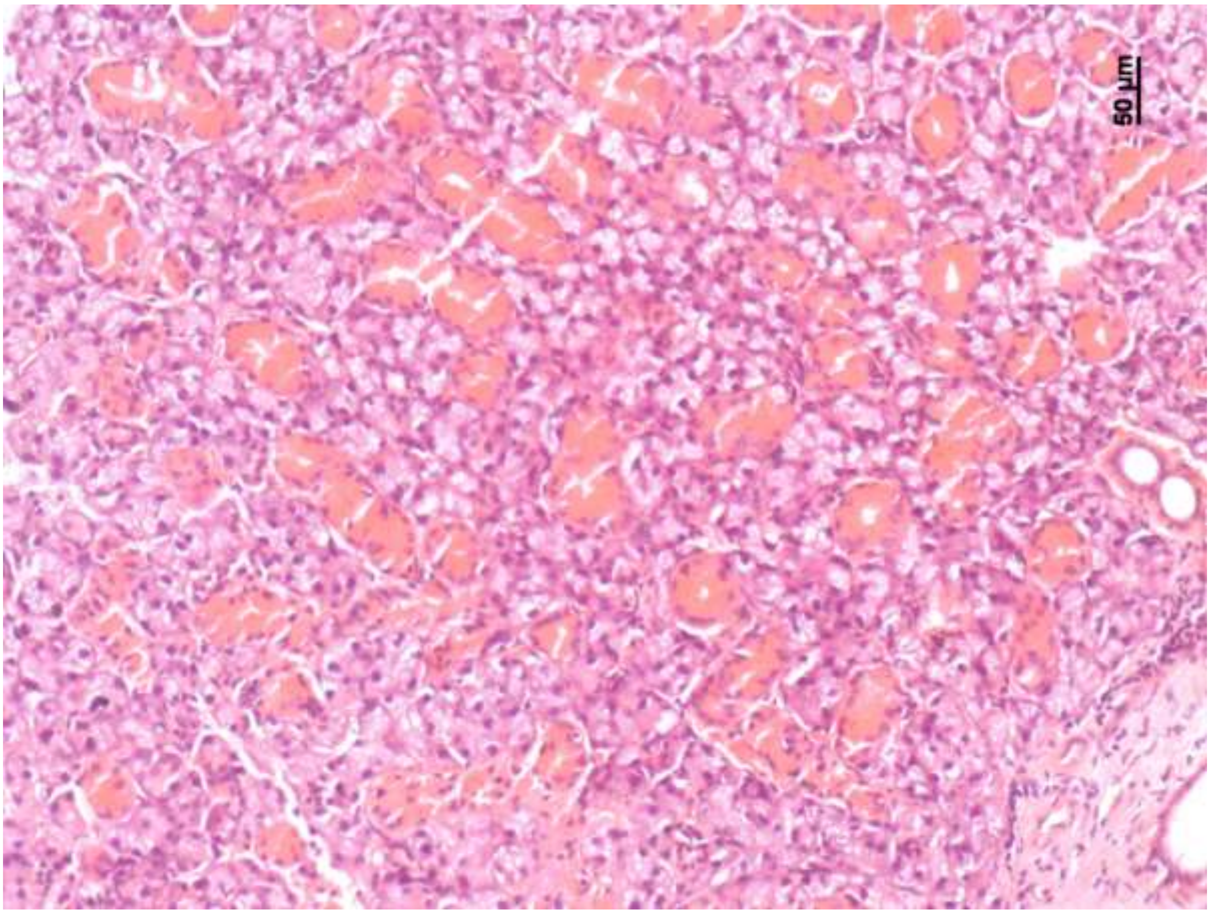


Figure 3: (a) and (b) represent Haematoxylin and Eosin (H&E) staining from a control and HFD3 liver. The arrows marked cytoplasmic vacuoles.

Figure 3A

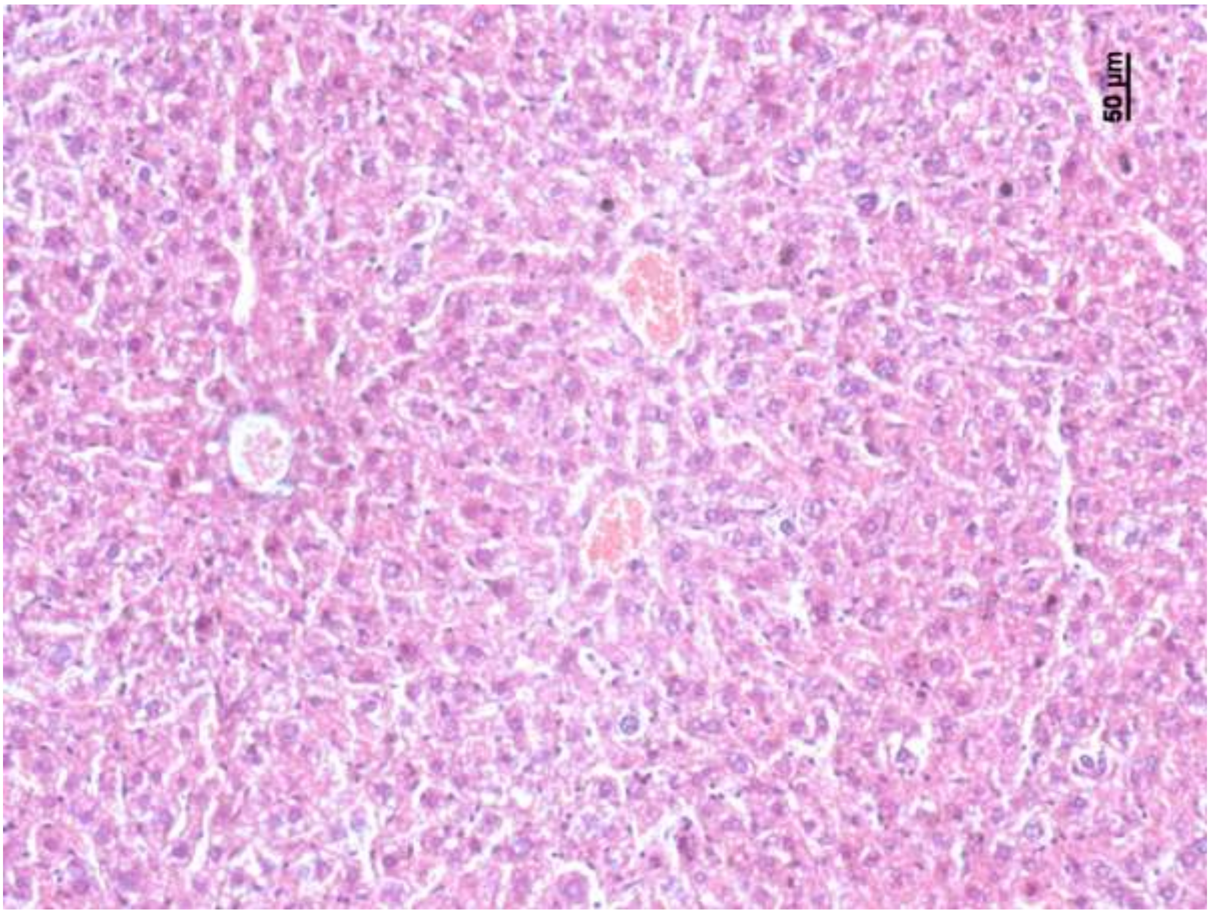


Figure 3B

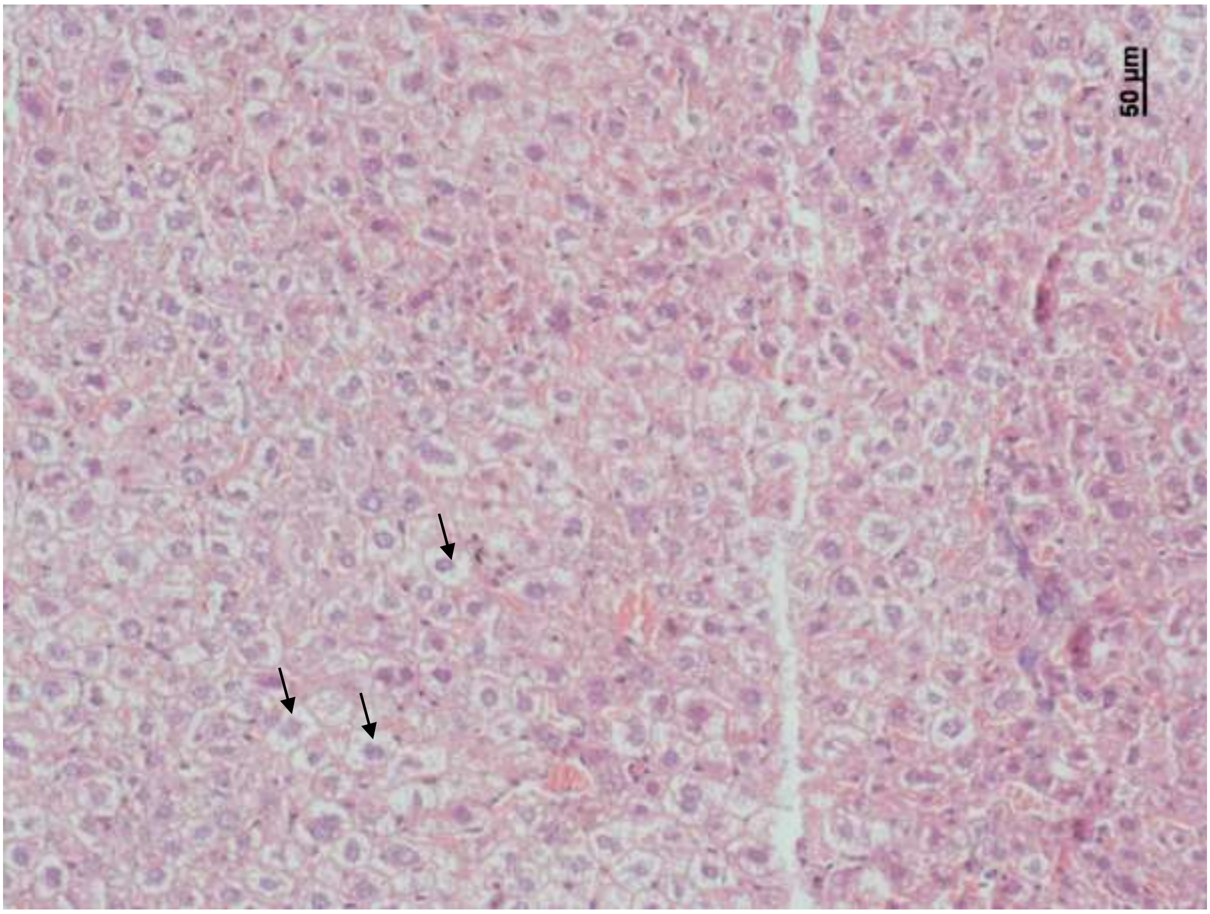


Figure 4: (a) and (b) represent Oil Red O staining in submandibular gland from controls after 3 month and HFD3 respectively. (c) and (d) represent a liver Oil Red O staining from controls after 3 month and HFD3 respectively.

Figure 4A

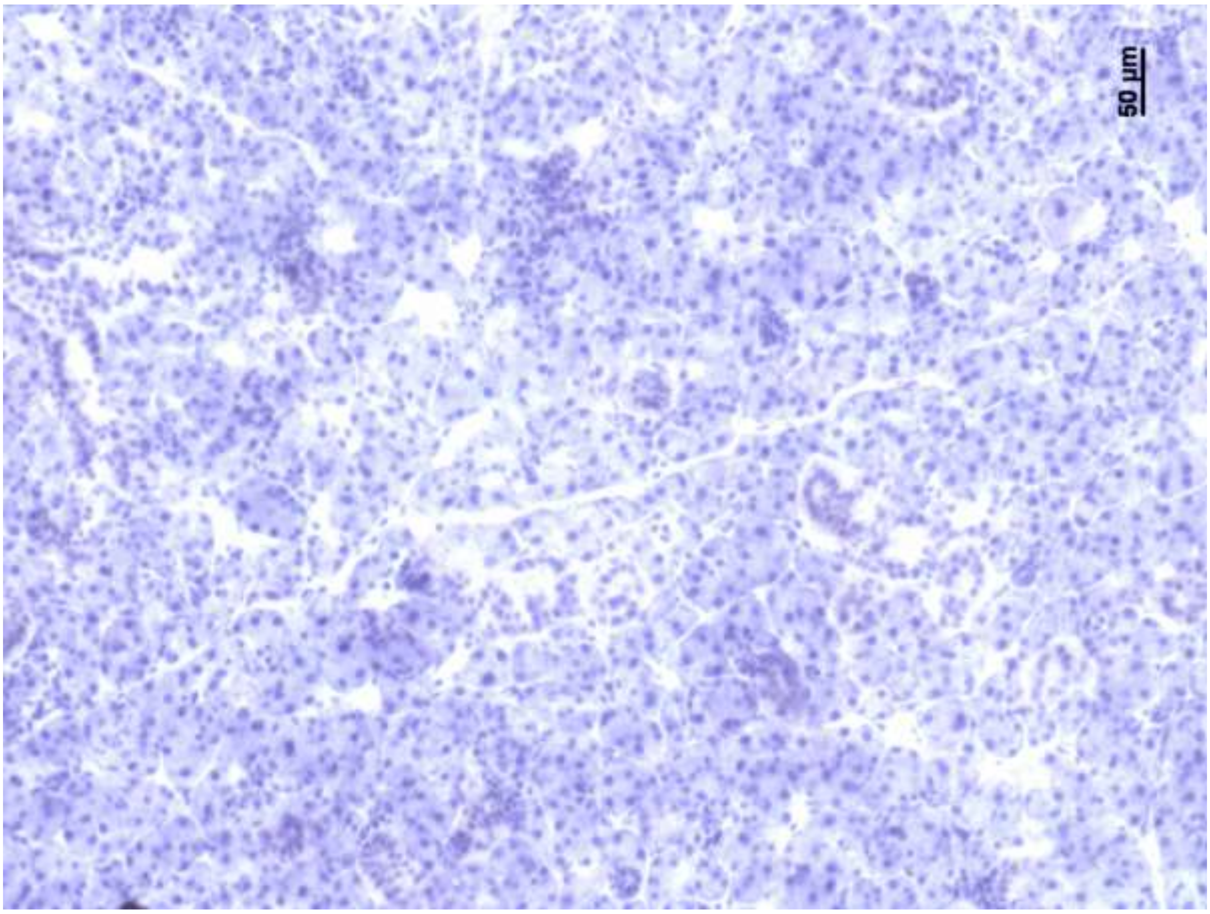


Figure 4B

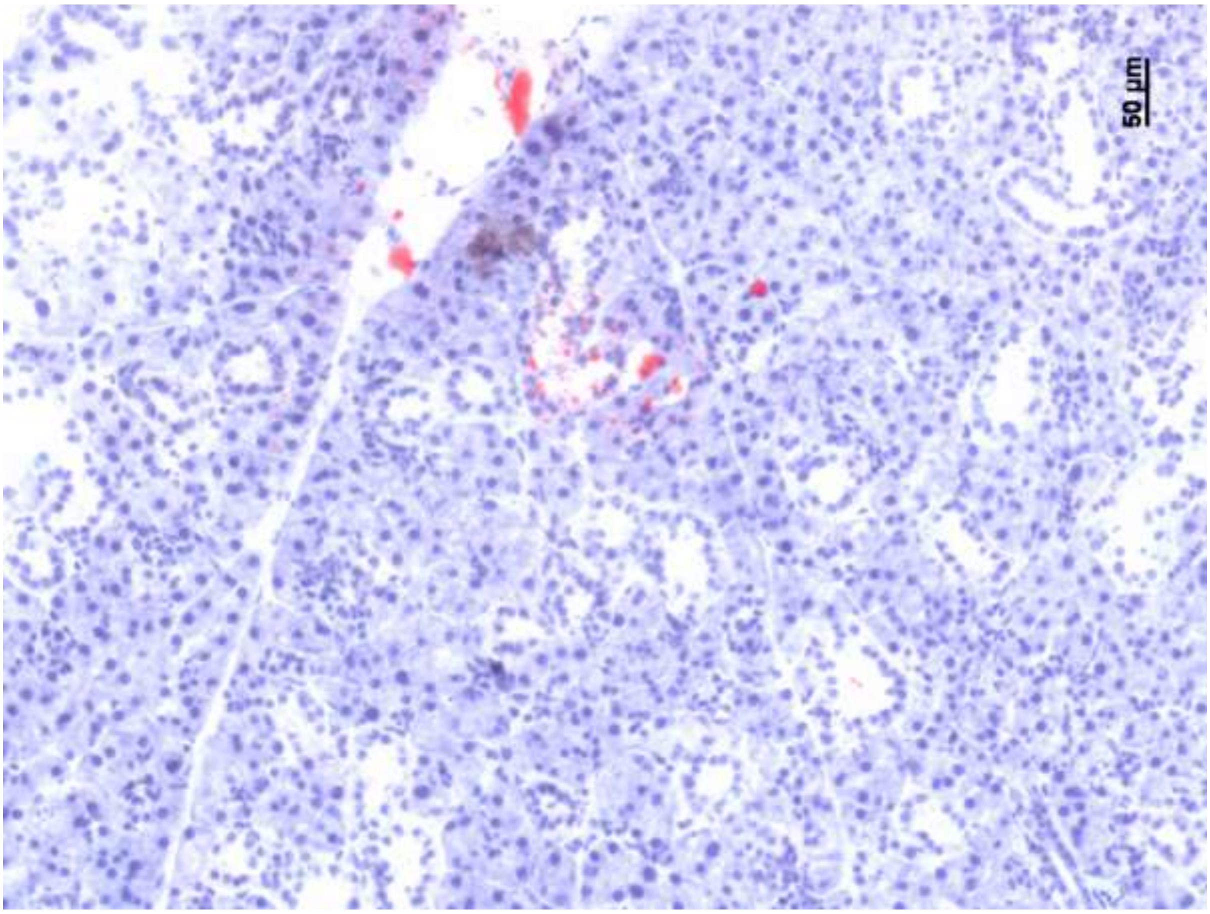


Figure 4C

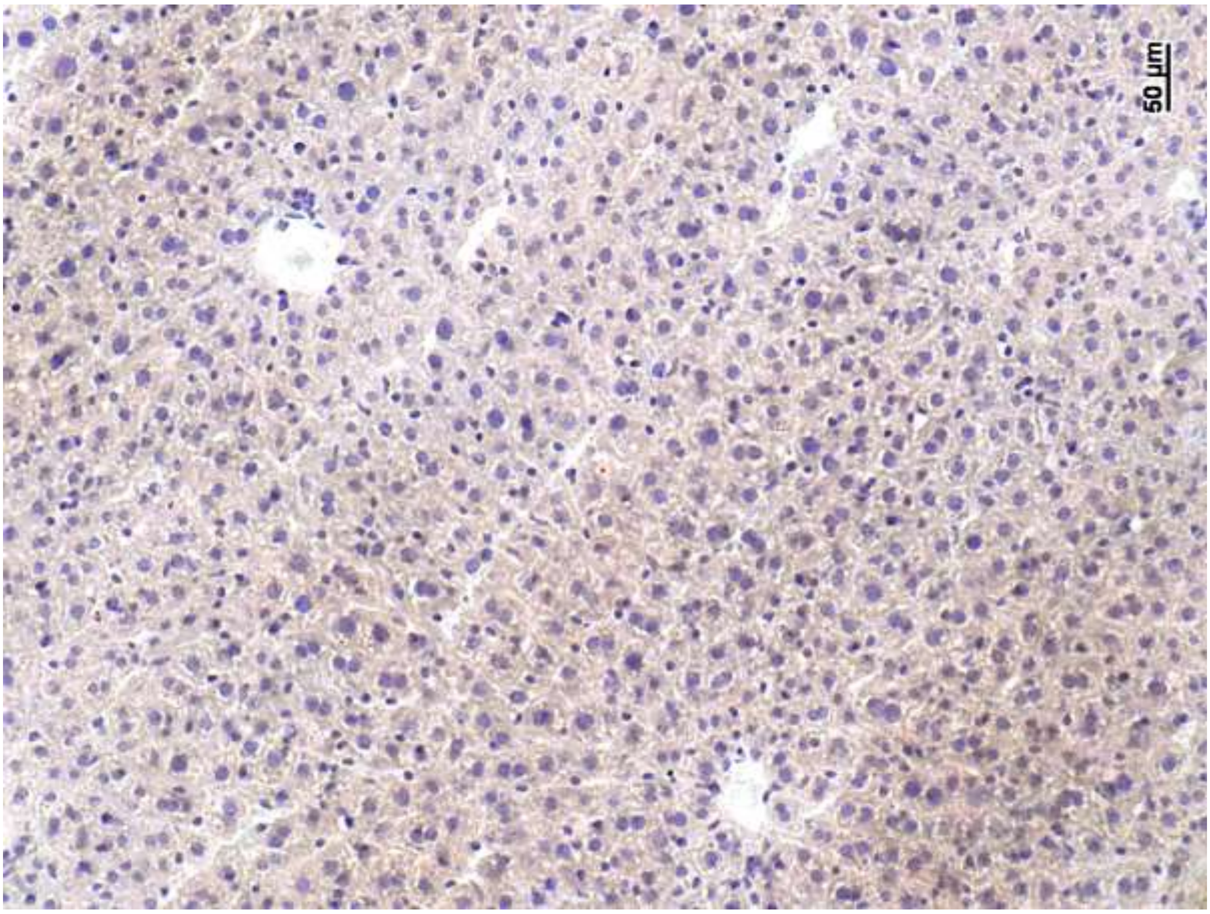


Figure 4D

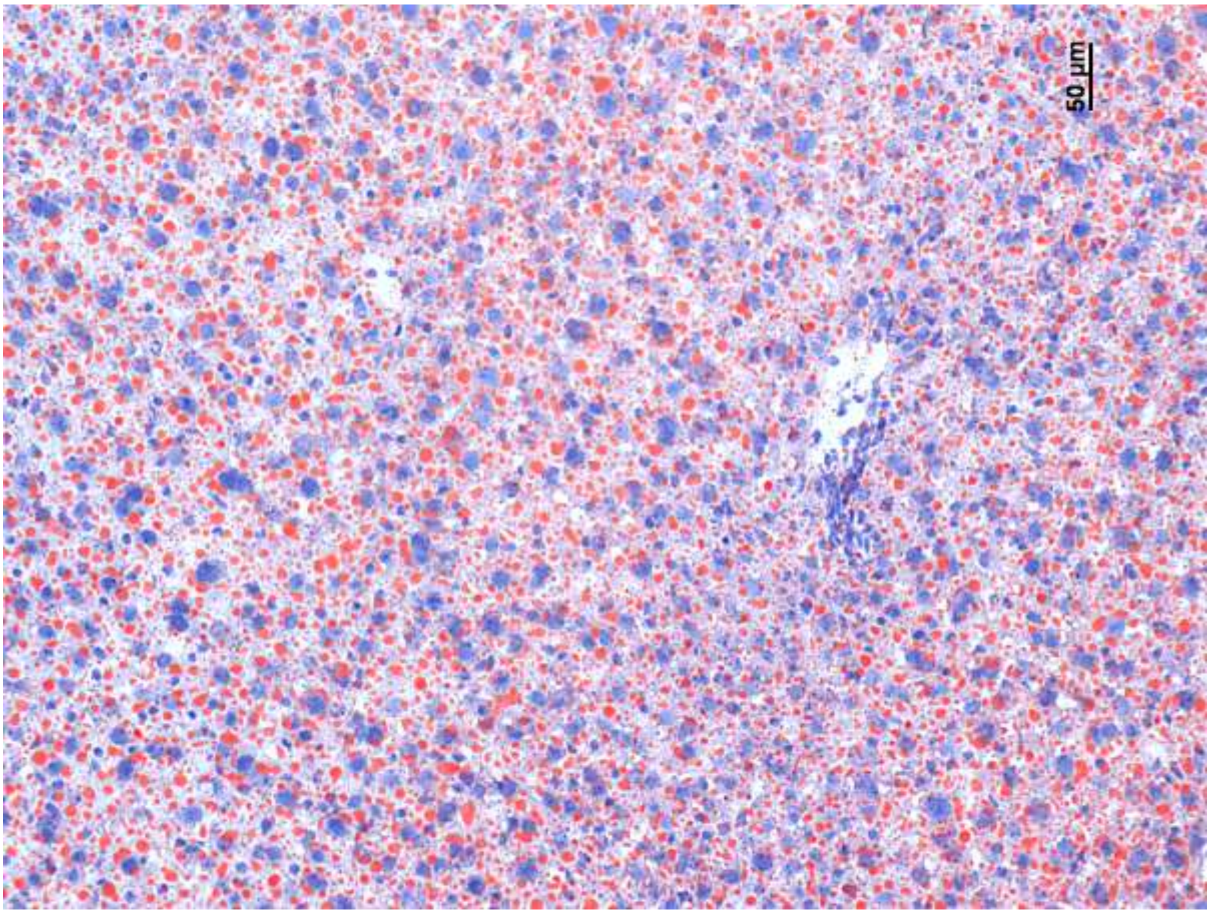
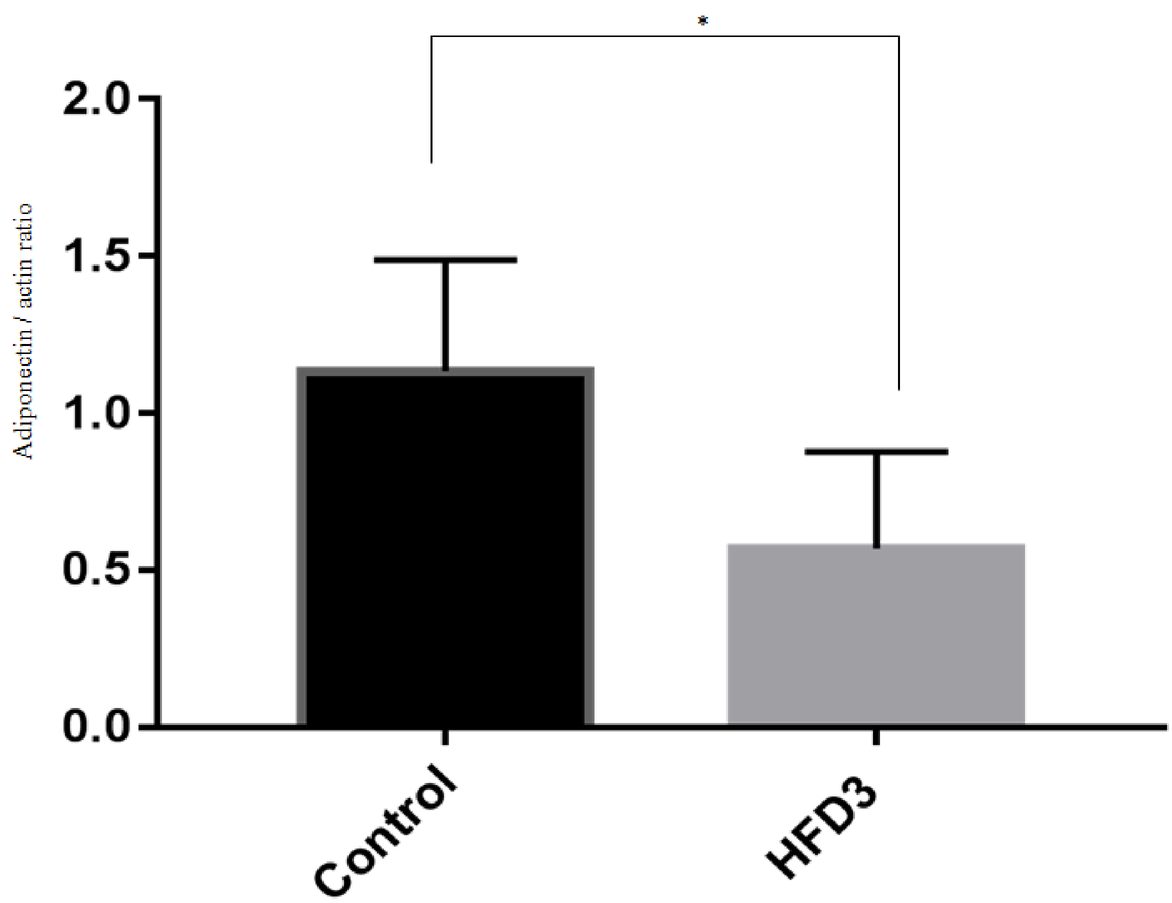


Figure 5: (a) show adiponectin as a ratio of β-actin (*P<0.05). The bars represent the mean ± S.D. Beta actin (β-actin) was used as a loading control. (b) immunoblotting of adiponectin protein expression in submandibular glands of mice fed HFD for three month. Data show is representative of three experiments.

Figure 5A



*P<0.05

Figure 5B


control
control
control
control
HFD3
HFD3
HFD3
HFD3
HFD3
Adiponectin
β actin
30kDa
42kDa

Figure 6: (a) show LC3 ratio (LC3II/LC3I) (**P*<0.05). The bars represent the mean ± S.D. (b) immunoblotting of LC3 expression in submandibular glands of mice fed HFD for three month. Data show is representative of three experiments.

Figure 6A

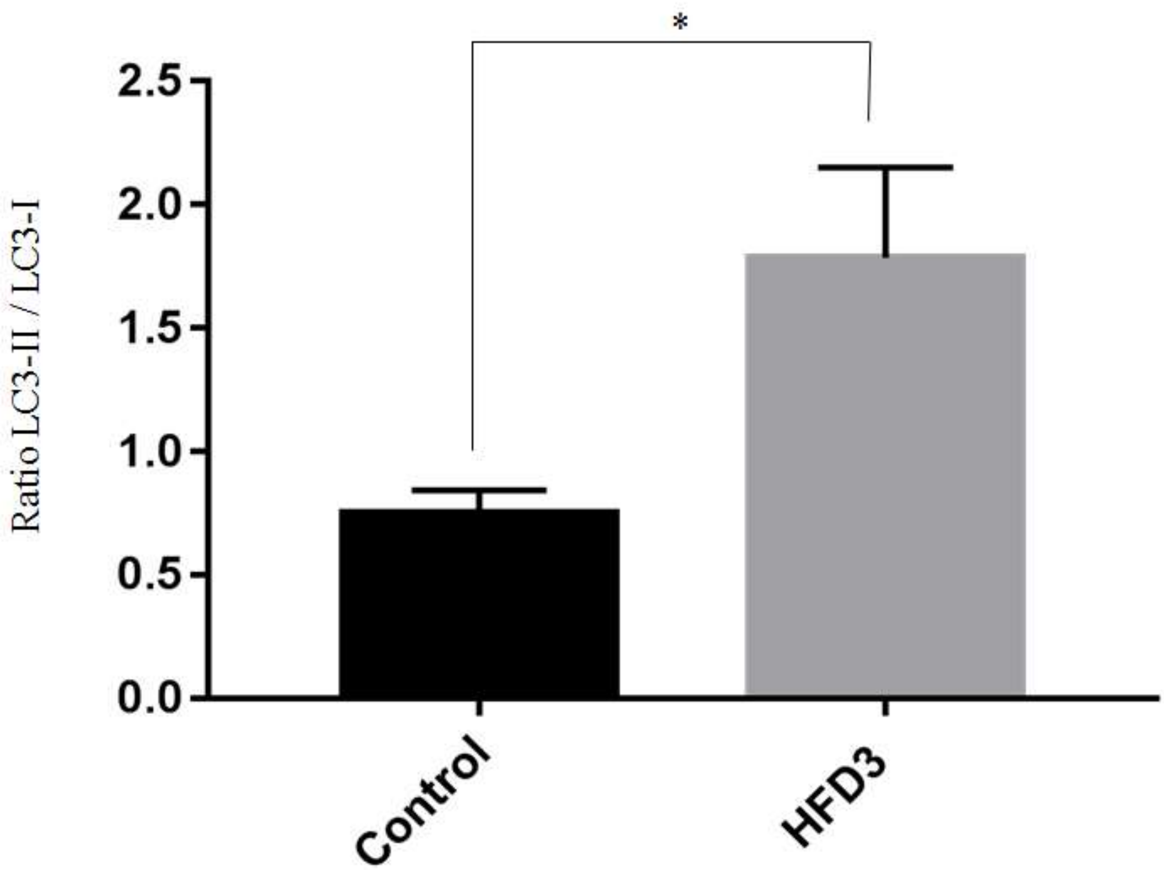


Figure 6B

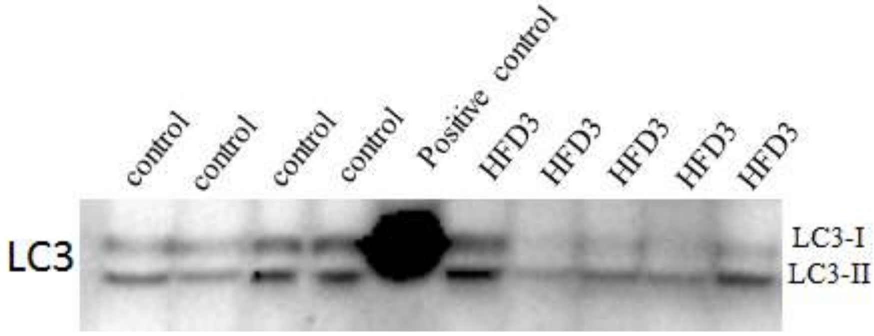

control
control
control
control
Positive control
HFD3
HFD3
HFD3
HFD3
HFD3
LC3
LC3-I
LC3-II

Figure 7: Immunoblotting of phosphor-4E-Bp1 protein for HFD1, HFD2 and HFD3 and respective controls.

Figure 7A

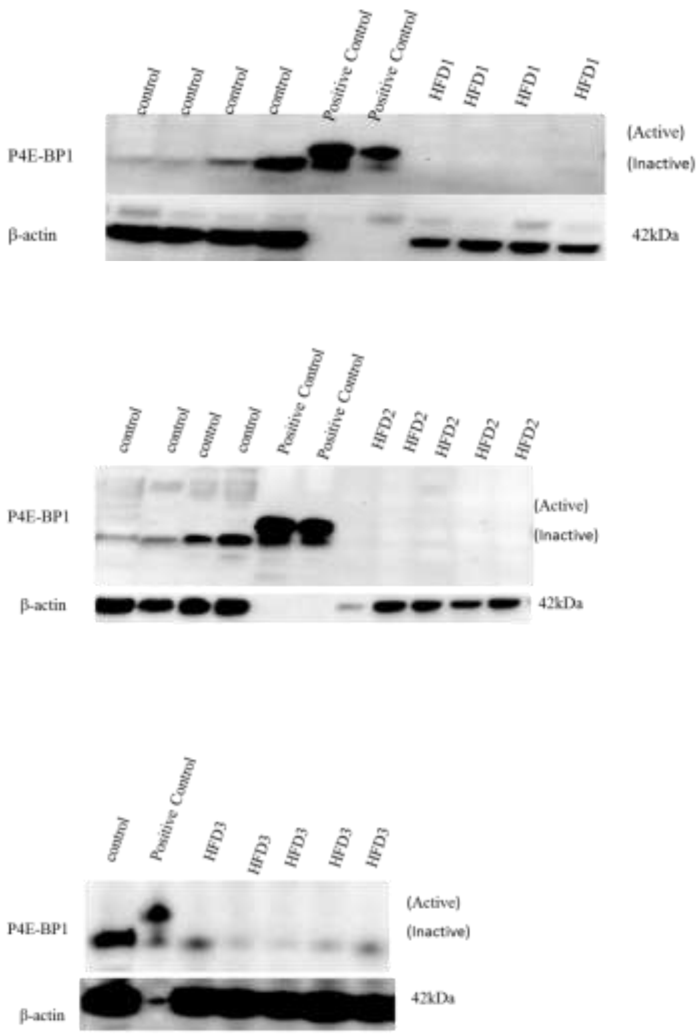


Figure8: (a) show tyrosine hydroxylase as a ratio of β-actin for mice fed HFD for two month and controls fed normal diet for the same period. (*P<0.05). The bars represent the mean ± S.D. Beta actin (β-actin) was used as a loading control. (b) immunoblotting of TH expression in submandibular glands of mice fed HFD for two month and controls.(c) show TH as a ratio of β-actin for mice fed HFD for three month and controls fed normal diet for the same period. (*P<0.05). The bars represent the mean ± S.D. (d) immunoblotting of TH expression in submandibular glands of mice fed HFD for three month and controls.

Figure 8A

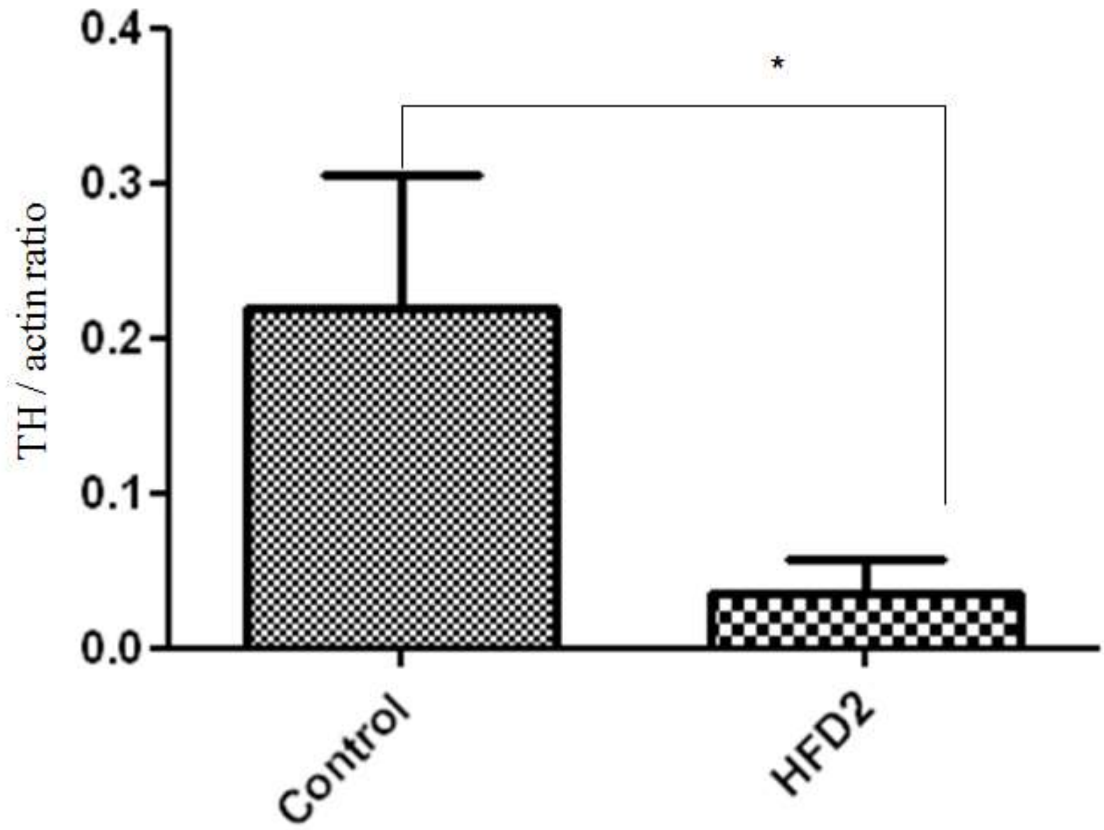


Figure 8B

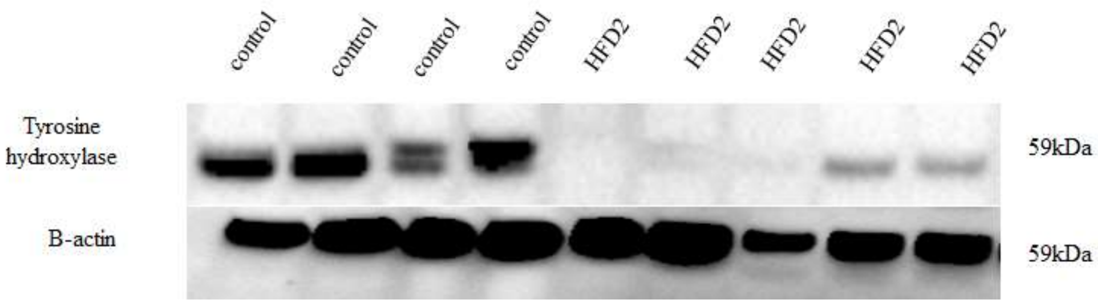

control
control
control
control
HFD2
HFD2
HFD2
HFD2
HFD2
Tyrosine hydroxylase
B-actin
59kDa
59kDa

Figure 8C

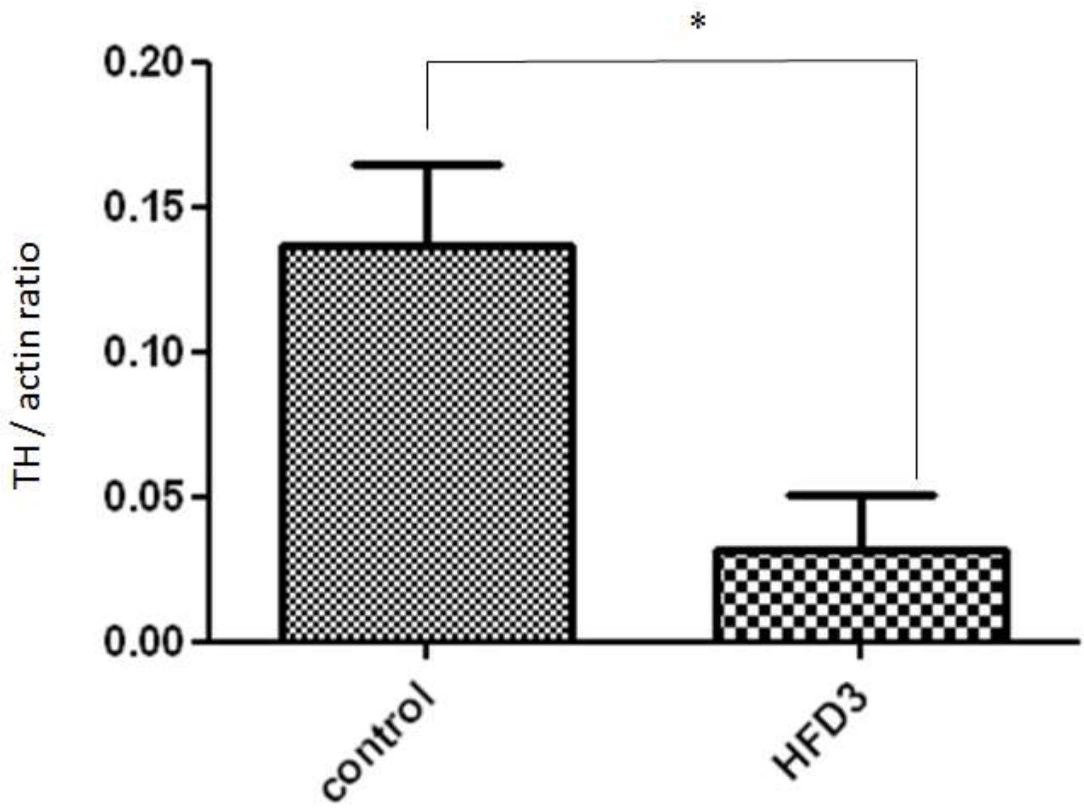


Figure 8D

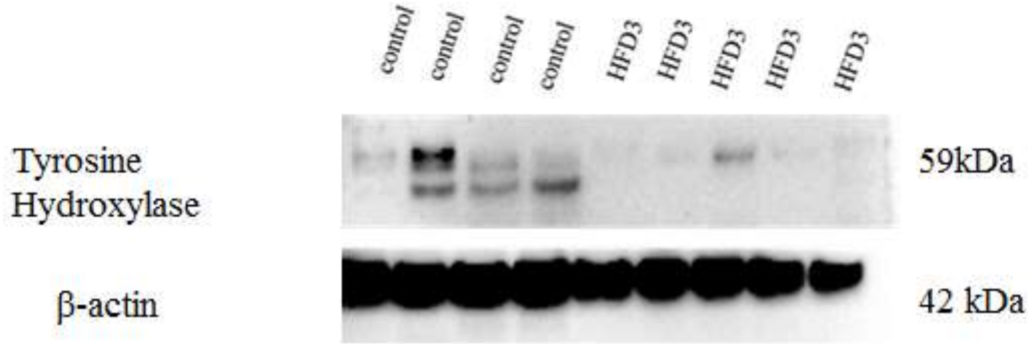

control
control
control
control
HFD3
HFD3
HFD3
HFD3
HFD3
Tyrosine
Hydroxylase
59kDa
β-actin
42 kDa

Figure 9: (a) Immunohistochemistry staining of tyrosine hydroxylase protein (brown) in submandibular glands in control (a) and HFD3 (b). A higher intensity of TH was found in controls compared with HFD fed ones.

Figure 9A

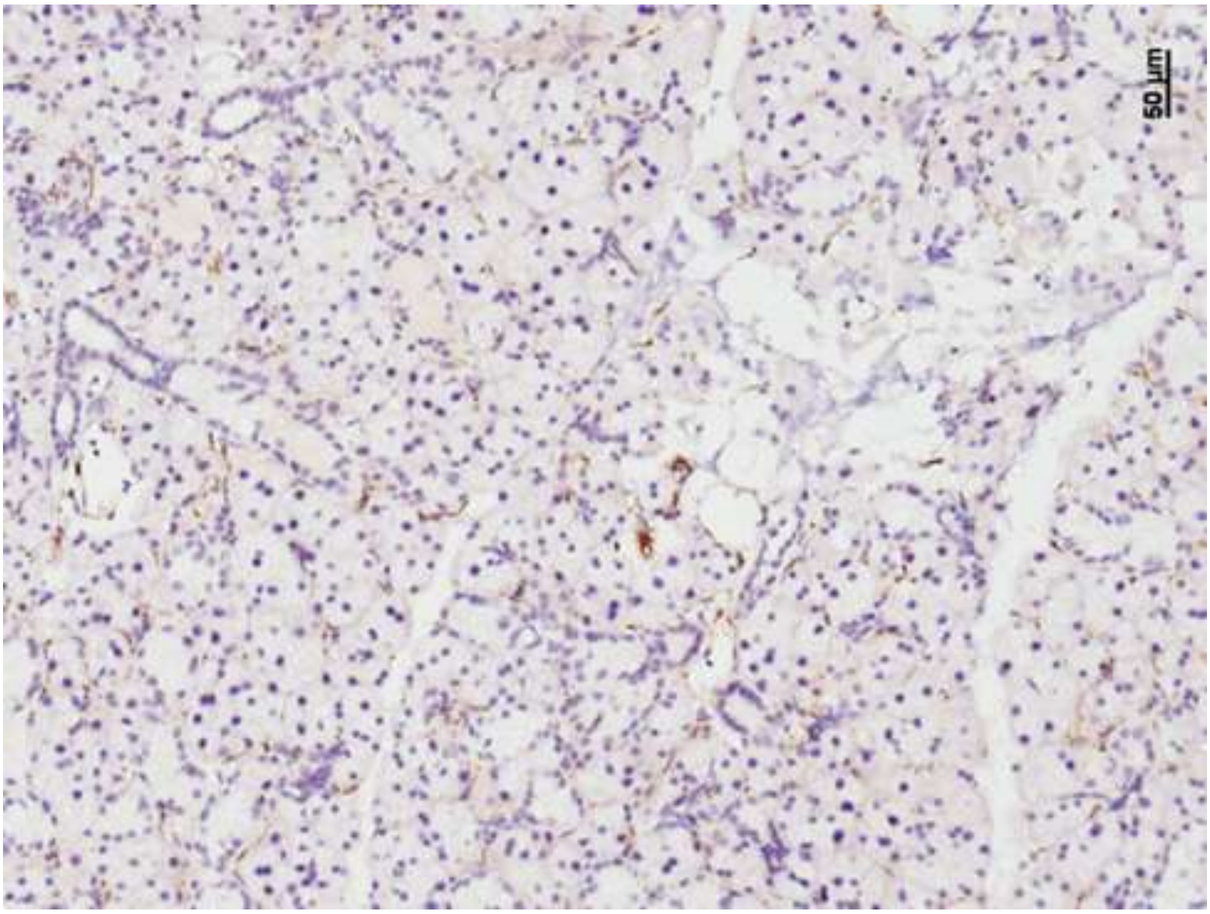


Figure 9B